C-glycosylxanthones in the *Asplenium adiantum-nigrum* Complex

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The polyphenolics of the Appalachian *Asplenium* complex (Smith & Harborne, 1971; Harborne et al., 1973) confirmed in all respects the concept of reticulate evolution in the species complex which had been proposed by Wagner (1954) on the basis of morphology and hybridization and chromosome studies. For example, *A. montanum* Willd. contained four *C*-glycosylxanthone compounds which were present in all hybrids containing the *A. montanum* genome. It has now been discovered that a parallel situation occurs in the chemistry of a group of European spleenworts, namely the *Asplenium adiantum-nigrum* complex. *Asplenium adiantum-nigrum* L. is an allotetraploid derived from *A. cuneifolium* Viv. and *A. onopteris* L. (Shivas, 1969). *Asplenium onopteris* is also a diploid progenitor of the tetraploid *A. balearicum* Shivas, the other diploid parent being *A. obovatum* Viv. (Shivas, 1969; Lovis et al., 1972). Thus, *A. onopteris* is analogous to *A. montanum*, the diploid ancestor of the tetraploids *A. bradleyi* D. C. Eaton and *A. pinnatifidum* Nutt. The analogy is further supported by the presence of *C*-glycosylxanthones in *A. onopteris*, *A. adiantum-nigrum*, and *A. balearicum (Fig. 1)*.

The European spleenworts are well known cytologically and morphologically (Lovis, 1977; Walker, 1979), but chemically they are virtually unknown. Several species have previously been surveyed for the presence of xanthones and proved negative: *A. adiantum-nigrum* (from Spain), *A. ruta-muraria* L., *A. septentrionale* (L.) Hoffm., *A. trichomanes* L., and the related species *Ceterach officinarum* DC. and *Phyllitis scolopendrium* (L.) Newm. (Smith & Harborne, 1971). A later reinvestigation of *A. adiantum-nigrum* indicated the presence of a xanthone-O-glycoside (Imperato, 1980). This particular compound had a 1,3,7,8-hydroxylation pattern in contrast to the 1,3,6,7-hydroxylation pattern of the *C*-glycosylxanthones found in the Appalachian spleenworts. It also had its sugar molecules attached by an oxygen linkage rather than by a direct carbon-carbon linkage as found in the *C*-glycosylxanthones. Imperato (1980) suggested that the absence of xanthones in the Spanish sample of Smith & Harborne (1971) may be due to phytogeographical factors.

A survey of ferns for the presence of *C*-glycosylxanthones is currently being undertaken at the New York Botanical Garden (Richardson, in press). The discovery of the *C*-glycosylxanthones mangiferin and isomangiferin in *Asplenium adiantum-nigrum* growing in the Enid A. Haupt Conservatory at NYBG led to a more intensive study of this plant and its related species. The possibility of geographic variation in xanthone production was examined by studying herbarium samples from almost the complete range of *A. adiantum-nigrum*. This led to the sampling of the diploid progenitors and ultimately to *A. balearicum*.

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MATERIALS AND METHODS

Apart from the *A. adiantum-nigrum* plant (NYBG 2066/76) which initiated the study, all experimental work was performed on dried material. Dr. T. G. Walker (Department of Plant Biology, University of Newcastle upon Tyne) kindly provided the sample of *A. balearicum*. The other samples were all in the herbarium at NYBG.

Asplenium adiantum-nigrum.—Scotland, *Nicholson* in 1881; England, *Taylor et al. 1731;* Switzerland, *Morthier* in 1879; Yugoslavia, *Richter* in 1910; Gran Canaria, *Cook 308;* Tenerife, *Kuntze* in 1888; Madeira, *Wilkes 44;* Lebanon, *Stutz 3045;* Punjab, *Stewart 7881;* Kashmir, *Stewart 17448* and *21784;* Caucasus, *Radde* Ex herb. horti. Petropolitani; South Africa, Ex. herb. Mt. Holyoke Seminary; Colorado, *Bethel 270;* Hawaii, *Degener H186* and *Degener & Wiebbe 3889*.

Asplenium cuneifolium.—Germany, *Missbach 5591* and *Luerssen 5706;* Austria, *Werderman & Meyer 232;* Hungary, *Richter 5592*.

A. onopteris.—Switzerland, *Leroy;* Italy, *St. Lager* in 1894; Dalmatia, *Ronniger* in 1926.

Small amounts of plant material were extracted in 80% methanol using a Polytron Homogenizer (Richardson, 1982). The extracts were initially screened for the presence of *C*-glycosylxanthones by one-dimensional chromatography on Whatman No. 1 paper in both water and 15% acetic acid. *C*-glycosylxanthones are revealed as orange spots under ultraviolet light (360 nm), turning fluorescent yellow with ammonia vapor. Extracts which proved positive were further analysed by two-dimensional chromatography on Whatman No. 3 paper in TBA (t-BuOH: HOAC: H_2O, 3:1:1) and HOAc (15% acetic acid). The *C*-glycosylxanthone spots were eluted in 80% methanol and co-chromatographed with mangiferin and isomangiferin (isolated from *Asplenium montanum, Bozeman & Radford 11552*, NYBG) in TBA, BAW, 15% HOAc, and H_2O. Acid hydrolysis of the extract (2N HCl, 1 hr, 100°C), followed by two-dimensional chromatography, was performed on the one fresh sample of *A. adiantum-nigrum*. For further details of the chromatography solvents and methods, see Harborne (1973) and Markham (1982).

RESULTS AND DISCUSSION

The two-dimensional chromatogram of the *A. adiantum-nigrum* from the conservatory revealed the presence of eleven polyphenolic compounds. Four were dark/yellow spots, typical of flavone and flavonol glycosides, with R_f values in TBA and HOAc as follows: 14,40; 17,51; 23,51; and 27,58. Seven were orange/yellow spots, typical of xanthones, with R_f values: 36,24; 37,49; 46,63; 20,72; 55,57; 55,68 and 70,64. The rutin marker was 39,68. After acid hydrolysis, the latter four xanthone spots disappeared and were replaced by a new xanthone spot at 48,26. This suggests that these latter four compounds were xanthone-O-glycosides, one of which was characterized by Imperato (1980). The other three xanthones were resistant to acid hydrolysis and were suspected to be *C*-glycosylxanthones. For two of the compounds this was confirmed by co-chromatography with mangiferin and isomangiferin, but the identity of the third *C*-glycosylxanthone remains unknown until larger quantitities of the compound become available for analysis.

Extracts from herbarium specimens of *A. adiantum-nigrum* produced either two or three xanthone spots on the two-dimensional chromatograms. In every case mangiferin and isomangiferin, and in some cases the unidentified *C*-glycosylxanthone, were readily visible and confirmed by co-chromatography. In no cases

were the four dark/yellow or the four xanthone-O-glycoside spots visible. This may be due to the small amounts of herbarium material available for analysis. It was usual to use only ca. 10–20 mg per chromatogram, compared to a whole plant sometimes used by Smith & Levin (1963). It is also possible that some of the missing compounds have been oxidized since some of the herbarium specimens date from the late nineteenth century.

The three samples of *A. onopteris* yielded two-dimensional chromatograms which were identical to those of the 'three-spotted' herbarium material of *A. adiantum-nigrum*. Each sample contained mangiferin, isomangiferin, and the same unknown *C*-glycosylxanthone. The four samples of *A. cuneifolium* produced blank two-dimensional chromatograms, even when repeated with comparatively large amounts of material. It can be stated firmly that *C*-glycosylxanthones were absent from all four samples. Only one specimen of *A. balearicum* was available, and the two-dimensional chromatogram indicated the presence of mangiferin, isomangiferin, and the same unknown *C*-glycosylxanthone, as well as a large dark/yellow spot at 32,55. However, there was insufficient material to verify the three xanthones by co-chromatography with the compounds from the other species. It would be interesting to examine larger amounts of *A. onopteris* and *A. balearicum* chemically in order to determine if they contain the xanthone-O-glycosides which occur in *A. adiantum-nigrum*.

Samples of *A. adiantum-nigrum* from throughout its range contained *C*-glycosylxanthones. This contrasts sharply with the earlier negative report by Smith & Harborne (1971). Possibly there is a genuine geographical variation in xanthone occurrence (this could be revealed by more intensive sampling). Alternately, the plant examined by Smith & Harborne (1971) may have been misidentified and may actually be *A. cuneifolium*. Most of the *A. cuneifolium* plants at NYBG were found to be misidentified as *A. adiantum-nigrum*, presumably due to the great similarity between the two species.

Asplenium onopteris is the first diploid European *Asplenium* species which has been found to contain *C*-glycosylxanthones. The compounds are absent from *A. cuneifolium*. *Asplenium obovatum* may or may not contain the compounds, and this cannot be predicted by the occurrence of the compounds in *A. balearicum*. If *A. obovatum* actually contains *C*-glycosylxanthones, then the compounds can be predicted to be present in both *A. billotii* Schultz, the autotetraploid derivative of *A. obovatum*, and *A. foreziense* Heribaud, another allotetraploid derived from *A. obovatum*. An investigation of the whole European *Asplenium* complex for *C*-glycosylxanthones would perhaps confirm the relationships established by cytological studies. The occurrence of *C*-glycosylxanthones in the European *Asplenium* complex also may help to relate them to the Appalachian spleenworts. Smith & Harborne (1971) mention that the ocurrence of xanthones in other *Asplenium* species may possibly suggest a relationship to *A. montanum*. Experimental hybrids of *A. onopteris* and *A. montanum* would reveal any chromosomes held in common. Finally, the occurrence of *C*-glycosylxanthones in the diploid *A. onopteris* and its tetraploid derivatives helps to confirm the suggestion by Smith & Levin (1963) that chemical investigations have significant potential for evolutionary studies involving polyploidy.

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